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IZUMENOLIDE—A NOVEL β-LACTAMASE INHIBITOR PRODUCED BY *MICROMONOSPORA*—III

THE STRUCTURE OF IZUMENOLIDE

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Abstract—Izumenolide, a β-lactamase inhibitor isolated from fermentations of *Micromonospora chalcea* subsp. *izumensis*, was shown by spectroscopic methods and chemical degradation to be a novel macrolide having structure **1**.

The β-lactam antibiotics constitute one of the major weapons against diverse bacterial infections by virtue of their potent antimicrobial activity and low toxicity. However, resistance arising from production of β-lactamases protects many otherwise susceptible pathogens. Hence, there has been a continuing search for agents to specifically inhibit these enzymes and thus serve as adjuncts in the therapeutic attack on β-lactamase producing organisms. This has recently led to the discovery and characterization of clavulanic acid and the olivanic acids, streptomycete-produced inhibitors that are themselves β-lactams. A review of the inhibition of β-lactamases has recently been published.[1]

We wish to report the structure of izumenolide (**1**), a potent inhibitor of β-lactamases that is not itself a β-lactam. The discovery, production by fermentation, isolation, preliminary chemical characterization, and biological characterization of izumenolide are being published elsewhere.[2,3]

RESULTS AND DISCUSSION

The isolation of **1** involved extraction into butanol at pH 2, back extraction into water at pH 7, precipitation from water by the addition of $Ca(NO_3)_2$, and regeneration of the Na salt. Thin-layer chromatography of the purest material obtained showed one major component and two minor components. Although efforts to further purify izumenolide on a preparative scale were not successful, this material was of adequate purity to determine the structure of the major component.

It is clear from the isolation scheme that **1** is acidic. Electrophoresis verified this, showing a high, constant mobility in the pH range 9.3 to 3.3, and undiminished mobility at the latter pH indicated that **1** is a strong acid.

The molecular weight of izumenolide was estimated by ultracentrifugation of the ammonium salt.[4] Values of 900 and 1300 were obtained in water and methanol, respectively. Failure of the inhibitor to dialyze through a membrane with a molecular-weight cutoff of 12,000 was inconsistent with this molecular weight range, but anomalous dialysis behavior of this sort has also been observed for the enzyme inhibitor, panosialin,[5] which has some gross chemical similarities to izumenolide. Elemental analysis of the sodium and ammonium salts of **1** showed that sulfur is a constituent of izumenolide and led, in conjunction with the molecular weight, to an approximate empirical formula of $C_{38-53}H_{69-103}O_{14-19}S_{3-4}M_{3-4}$ ($M=Na^+$ or NH_4^+).

The IR spectrum (strong peak at 1230 cm^{-1}) and the strongly acidic nature of **1** indicated that it may be a sulfate ester and this was confirmed by acid hydrolysis. Izumenolide is fairly stable to acid at room temperature, giving no change in 1 N HCl after 24 hr; however, all activity is lost after heating at 100° for 5 min. Heating at 100° in 6 N HCl for 1 hr gave inorganic sulfate, isolated as the barium salt, in good yield based on the elemental analysis.

The other functionality present in izumenolide was largely established by spectroscopic methods on the intact agent. An α,β-unsaturated ester with the *E*-configuration was indicated by peaks in the IR spectrum at 1711 and 1646 cm^{-1}, by a max at 214 nm in the UV spectrum, and by absorption in the ^1H-NMR spectrum at 5.77 (d, J = 15.7 Hz) and 6.83 ppm (d, t, J = 15.5, 6.8 Hz) and in the ^{13}C-NMR spectrum (Table 1) at 122.5, 151.0 and 167.9. Comparison of the intensity of the UV absorption of the sodium salt ($E^{1\%}_{1cm} = 95$) to that of methyl crotonate (UV max at 212 nm, log ε 4.16) indicated a molecular weight for **1** (*ca* 1500) that is somewhat higher than the values obtained by ultracentrifugation and distinctly higher than that corresponding to the structure ultimately derived (Na salt: MW 941). An additional double bond in **1** was indicated by peaks in the ^1H-NMR spectrum at 5.38 ppm and in the ^{13}C-NMR spectrum at 125.5 and 132.5 ppm. The position of this double bond was established by ozonolysis (Me_2S work-up[6]) giving acetaldehyde, isolated as the dinitrophenylhydrazone. The *E*-configuration was assigned from the chemical shifts of the olefinic carbons and from the chemical shift (18.2 ppm) of the terminal Me group (expected at 18 ppm in the *E*-olefin and at 13 ppm in the *Z*-olefin).[7]

Izumenolide is rather stable to base, but gives the chromatographically homogeneous substance **2** after 5 hr at 100° in 1 N NaOH. The ^{13}C-NMR spectrum of **2** has a peak at 68.5 ppm assigned to a hydroxylated carbon that correlates with a peak at 73.3 ppm in the spectrum of **1**. A carboxylate group (IR: 1570 and 1420 cm^{-1}) is also present in **2**, establishing that the

Table 1. ^{13}C-NMR spectrum of izumenolide (a)

Chemical Shift (b)	Assignment (c)	Chemical Shift	Assignment
(12.7)	40'	40.2 t	16,30
18.1 q	40	73.2 d	29
25.8 t, 26.0 t	13,19,27,33	78.0 d	15,17,31
		122.5 d	2
(27.7)	37'	(124.5)	39'
29.1	5	125.5 d	39
30.2	6,36	(131.6)	38'
30.7	7~12,20~26,34~35	132.5 d	38
(33.1)	4'''	150.7 d	3
33.5	4,37	(151.0)	3'''
35.5, 35.9	14,18,28,32	167.9 s	1
		(172.5)s	1''

(a) Ammonium salt in CD_3OD. (b) Chemical shifts are in ppm downfield from TMS. CD_3OD (49.0 ppm) was used as an internal reference. Multiplicities were determined by single-frequency off-resonance decoupling. Weak peaks due to minor components are in parentheses. (c) The numbering system is shown in structure 1. Primed numbers are assigned to the 38,39-Z isomer, the double primed number to the 2,3-dihydro component, and the triple primed numbers to the base hydrolysis product.

ester function in **1** is lactonic. A peak at 78.0 ppm in the spectrum of **1**, attributed to the sulfated carbons, is unchanged in the spectrum of **2**. The ^{13}C-NMR spectrum of izumenolide contains a weak peak at 151.0 ppm, that may be due to a trace of **2** produced during isolation. In addition to the peaks discussed above, the ^{13}C-NMR and ^{1}H-NMR spectra of izumenolide have intense peaks at 30.7 and 1.23 ppm, respectively, indicative of substantial $-(CH_2)_n-$ regions.

The exact nature of the carbon skeleton of **1** was determined by the degradative sequence shown in Chart 1. Hydrogenation of **1** yielded a derivative (**3**) that retained β-lactamase-inhibiting activity. Since tlc of **3** showed minor components with the same mobilities as those that contaminate **1**, these minor components are not double-bond isomers or analogs with more or less unsaturation. It was thought that treatment of **3** with $LiEt_3BH$ might reductively remove the sulfate esters by analogy with the corresponding reduction of sulfonate esters;[8,9] they were, however, inert (no reaction after one week) under the conditions used. Only slow reductive opening of the lactone to the diol **4** occurred.[10] Acid hydrolysis of **4**, followed by tosylation of the resulting alcohol **5**, gave the pentatosylate **6**. Treatment of **6** with $LiEt_3BH$[8] then gave a crystalline hydrocarbon that was easily identified as n-tetracontane (**7**) from the mass spectrum and by comparison with authentic material. The empirical formula of **1** (Na-salt) is thus established as $C_{40}H_{71}O_{14}S_3Na_3$. Agreement with the elemental analysis[2] is surprisingly good, considering the

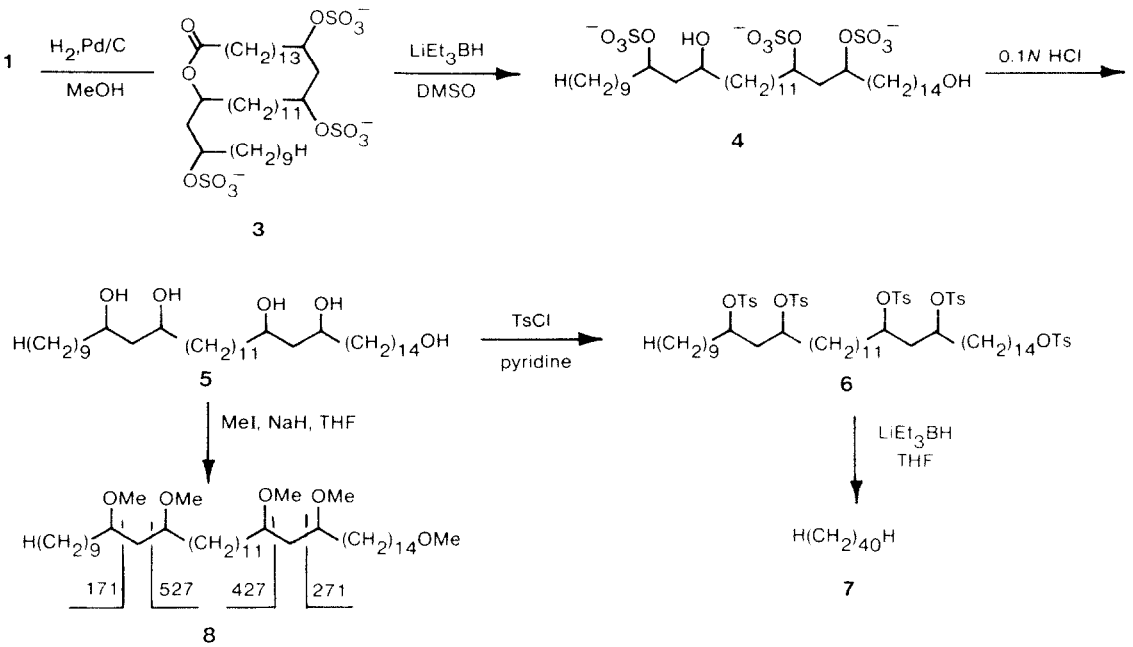
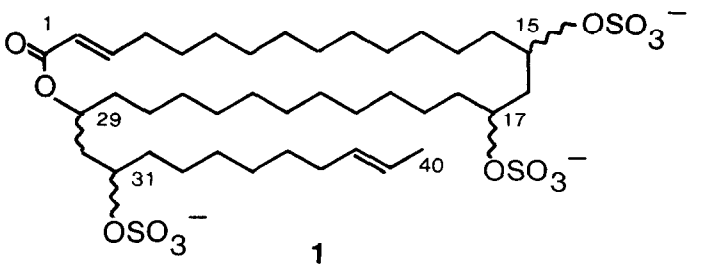


Chart 1.



1

extraneous components observed by tlc and the brevity of the isolation scheme.

Methylation of the pentaol **5** with methyl iodide gave pentamethyl ether **8**. The mass spectrum of this compound served to locate the oxygenated carbons in **1**. Major fragments were observed at *m/z* 171, 271, 427, and 527; these uniquely establish oxygenation at C-15, C-17, C-29, and C-31 (in addition to C-1) as shown in structure **8**.

The position of closure of the lactone was established by the sequence in Chart 2. Methylation of the diol trisulfate **4** gave the dimethylether trisulfate **9**. Hydrolysis to the dimethylether triol **10**, followed by tosylation and reduction of the resulting tosylate **11** with $LiEt_3BH$, gave the dimethyl ether **12**. Mass spectral fragments at *m/z* 199 and 467, as shown in structure **12**, established oxygenation at C-29 (in addition to C-1). Izumenolide is thus a 30-membered lactone and the full structure, exclusive of configuration of C-15, C-17, C-29 and C-31, is as shown by structure **1**.

The sequence shown in Chart 3 was also applied to izumenolide. Hydrolytic removal of the sulfate groups from the base hydrolysis product **2** gave a tetraol that was converted to the trimethylsilyl derivative **13** and examined by mass spectroscopy. Fragmentation to give ions with *m/z* 227, 399, 599 and 771 is consistent with the structure proposed for **1**. However, additional ions are observed at *m/z* 401 and 773. These apparently arise from a component in which the C-2, C-3 linkage is saturated. This is consistent with the UV spectrum of izumenolide which gave absorption at 212 nm that was somewhat weaker than that expected for an α,β-unsaturated ester. The ^{13}C-NMR spectrum of izumenolide has a weak peak at 172.5 ppm that is reasonably assigned to the lactone carbonyl of 2,3-dihydro izumenolide.

The ^{13}C-NMR spectrum of izumenolide also indicates that there is a minor component in which the 38,39-double bond has the *Z*-configuration. Weak peaks at 27.7, 131.6, 124.5 and 12.7 ppm are assigned, respectively, to C-37, C-38, C-39 and C-40 in this

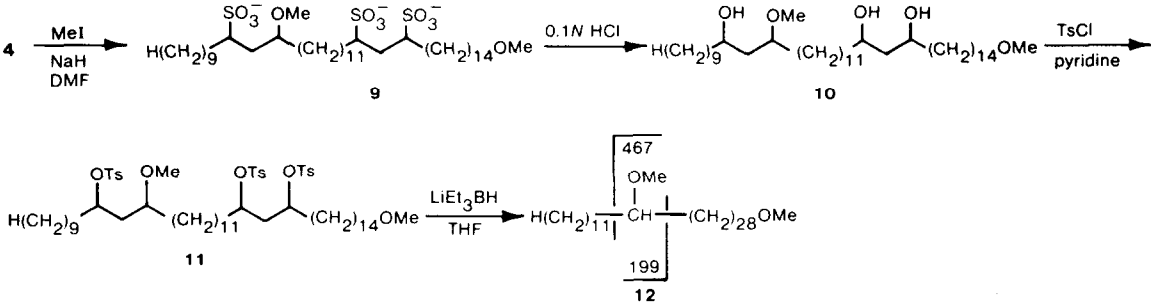
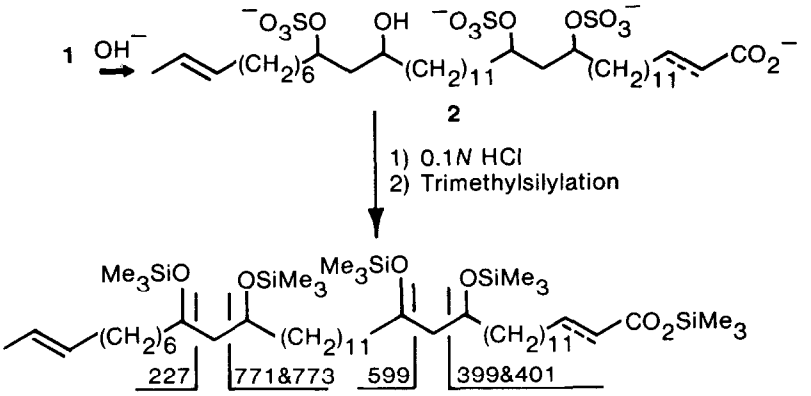


Chart 2.



13a α, ß-unsaturated
13b α,ß-saturated

Chart 3.

isomer.[7] The spectrum also has weak peaks at 33.1 and 151.0 ppm that could be due to C-4 and C-3 of base-hydrolysis product **2**, known (tlc and IR spectrum) to contaminate izumenolide to a small extent (as one might expect, considering the isolation).

As mentioned above, the 2,3-dihydro and the 38,39-*Z* components (and presumed combinations) would not be expected to be chromatographically distinct in the system used (Experimental, hydrogenation of **1**). Since intermediates in the degradation were chromatographically purified, and minor components discarded, it is not possible to provide any informed speculation about the minor components that are chromatographically distinct.

To the best of our knowledge, izumenolide is the first macrolide reported to have sulfate ester groups. The structure is unusually simple, having no branching in the carbon skeleton. The substitution pattern, in which only odd-numbered carbons are oxygenated, is consistent with straight-forward biosynthesis from acetate.

It was observed in the course of the degradation that the β-lactamase inhibitory activity was completely retained by the hydrogenation product **3**, and was partially retained by the other degradation products (**2**, **4** and **9**) in which the sulfate esters were still intact. This is further discussed in Ref. 3.

EXPERIMENTAL

NMR spectra were recorded on Varian Associates XL-100-15 and Jeol Ltd. FX60Q spectrometers; chemical shifts are given in ppm (δ) downfield from internal Me_4Si or $Me_3SiCD_2CD_2CO_2Na$. Mass spectra were determined on an AEI MS-902 double-focusing mass spectrometer.

Tlc was done on Merck silica gel 60 plates eluting with 2-BuOH–HOAc–H_2O, 3:1:1 (System A); $CHCl_3$–MeOH, 9:1 (System B); or as specified. Phosphomolybdic acid-H_2SO_4 was used for detection unless noted otherwise. The various sulfate esters were used as their sodium salts unless otherwise specified.

Acid hydrolysis of **1**. An 11.3-mg sample of **1** was dissolved in 0.5 mL of MeOH and 0.5 mL of 6 N HCl and the soln heated in an open tube at 100° for 1 hr. The resulting mixture was concentrated to dryness and the residue distributed in n-BuOH–NH_4OHaq. The aqueous phase was acidified with excess conc HCl and treated with $Ba(OH)_2$, giving 7.4 mg of $BaSO_4$ (calculated: 7.9 mg).

Base hydrolysis of **1**. A soln of 105 mg of **1** in 2 mL 1 N NaOH was heated under N_2 in a sealed tube at 100° for 5 hr. The resulting soln was neutralized (H_2SO_4), saturated with Na_2SO_4 and extracted with several portions of n-PrOH that had been equilibrated with warm, sat Na_2SO_4aq. The extract was concentrated *in vacuo* and the residue triturated with MeOH. The mixture was filtered and the filtrate mixed with EtOAc and concentrated *in vacuo*, giving 101 mg of **2**: IR (KBr) 1570 and 1420 ($-CO_2^-$) and 1230 cm^{-1} ($-OSO_3^-$), tlc (System A) R_f 0.47; ^{13}C-NMR (ND_4^+ salt in CD_3OD) δ 169.9 (C-1), 151.1 (C-3), 132.5 (C-38), 125.4 (C-39), 122.3 (C-2), 78.0 (C-15, C-17 & C-31), 68.4 (C-29), 18.1 (C-40), 12.8 [w, (Z)–CH=CH–$\underline{C}H_3$]. (Found: C, 48.97; H, 7.42; S, 9.94; Na, 9.46. Calc. for $C_{40}H_{72}O_{15}S_3Na_4$: C, 48.97; H, 7.40; S, 9.80; Na, 9.37%).

Hydrogenation of **1**. A soln of 0.95 g of **1** in MeOH was treated at 1 atm with H_2 and 10% Pd/C in three 0.5-g portions, resulting in the uptake of 41 mL of H_2 (exclusive of that taken up by the catalyst). (Calc. 45 mL for an equivalent wt. of 470.6). The mixture was filtered washing with water and the filtrate concentrated *in vacuo*. The residue was converted to a dry powder by dissolving in MeOH, adding MeCN, and concentrating *in vacuo*, giving 0·82 g of **3** (Na salt): tlc (System A) R_f 0.11 (tr), 0.14 (s), 0.18 (w).

A sample of **3** was converted to the ammonium salt by passage in MeOH-H_2O (1:1) through a column of Dowex 50W-X2 (NH_4^+). The ammonium salt was converted to a dry, deliquescent powder as above: UV (water) weak end absorption; ^{13}C-NMR (CD_3OD) δ 175.2 (C-1), 78.0 (C-15, C-17 & C-31), 72.9 (C-29), 14.5 (C-40). (Found: C, 50.74; H, 9.01; N, 5.01; S, 10.58, Ash 1.34. Calc. for $C_{40}H_{88}N_3O_{14}S_3$: C, 51.64; H, 9.43; N, 4.52; S, 10.34%).

Lithium triethylborohydride reduction of **3**. A carefully dried 165-mg sample of **3** was dissolved under Ar in 3.3 mL of dry DMSO, and 2 mL of 1 M $LiEt_3BH$ in THF was added. After 72 hr at room temp, sat Na_2SO_4aq was added. The product was extracted into n-PrOH and the extract concentrated to dryness. The residue was triturated with MeOH and the resulting filtrate mixed with MeCN and concentrated *in vacuo*, giving 225 mg of solid. This was dissolved in water and applied to a 80-ml column of Amberlite XAD-2 in water. The column was washed with water and then the product was eluted with MeOH. The MeOH eluate was concentrated *in vacuo* and the residue converted to a powder by concentration of a MeOH–EtOAc mixture. This gave 130 mg of **4** as a deliquescent powder: tlc (System A) indistinguishable from that of **2**; IR (KBr) 1236 cm^{-1} ($-OSO_3^-$). (Found: C, 50.70; H, 8.67; S, 8.52; Na, 8.34. Calc. for $C_{40}H_{79}O_{14}S_3Na$: C, 50.61; H, 8.39; S, 10.13; Na, 7.27%).

The product was converted to the ammonium salt as described for **3**: ^{13}C-NMR (CD_3OD) δ 78.0 (C-15, C-17 & C-31), 68.5 (C-29), 63.0 (C-1), 14.5 (C-40).

Acid hydrolysis of **4**. A soln of 88.5 mg of the ammonium salt of **4** in 10 mL 0.1 N HCl was heated on a steam bath for 90 min. The resulting mixture was cooled and extracted with several portions of the lower phase of MeOH–$CHCl_3$–H_2O, 1:1:1. The extract was concentrated *in vacuo* and the residue (58 mg) dissolved in $CHCl_3$–MeOH (9:1) and filtered (hot) through a 1-mL column of silica gel to remove polar impurities. Concentration of the effluent gave 42.5 mg of **5**: tlc (System B) 0.33 (w), 0.39 (w), 0.45 (s), 0.53 (w); mass spectrum of trimethylsilylated derivative, m/z 912 ($M^+ - Me_3SiOH$).

Tosylation of **5**. A soln of 42.5 mg of **5** and 191 mg TsCl in 1 mL dry pyridine was left at 5° for 17 hr. Work up and tlc (*vide infra*) showed incomplete reaction. The product was retreated as above but for 15 hr at room temp. The resulting soln was diluted with water and extracted with ether. The extract was washed with sat $NaHCO_3$aq, water, sat NaClaq, dried ($MgSO_4$) and concentrated *in vacuo*, giving 45 mg residue. This was purified by preparative tlc on silica gel, eluting with $CHCl_3$. The major fluorescence-quenching band, R_f 0.5 ~ 0.6, was collected giving 17.9 mg of **6** as a colorless oil: IR ($CHCl_3$) 3030, 3010 & 1600 (Ar), 1360 & 1176 cm^{-1} ($ROSO_2Ar$); ^{1}H-NMR ($CDCl_3$) δ 7.77 & 7.33 (10 H, AB quartet, J = 8.3 Hz, ArH), 4.51 (4 H, quintet, J = 5.8 Hz, H–C–OSO_2Ar), 4.02 (2 H, t, J = 6.4 Hz, $-CH_2OSO_2Ar$).

Lithium triethylborohydride reduction of **6**. The pentatosylate **6** was dissolved under Ar in 0.5 mL of 1 M $LiEt_3BH$ in THF, giving a clear soln that gelled in a few min. After 19 hr at room temp, MeOH was added followed by water. The mixture was extracted with $CHCl_3$ and the extract concentrated *in vacuo*. The residue in hot heptane was filtered through a small column of silica gel. Concentration of the effluent gave 5.4 mg of **7** as a crystalline solid: m.p. 80.0 ~ 81.0°; mmp with n-tetracontane 80 ~ 81.5°; IR (neat crystalline film) and mass spectra indistinguishable from those of n-tetracontane.

Methylation of **5**. A mixture of 28 mg of **5**, 228 mg of NaH, and 0.5 mL of MeI in 5 mL of THF was stirred at room temp under N_2 for 21 hr. Excess NaH was destroyed with MeOH and the mixture was concentrated *in vacuo*. The residue was mixed with water, acidified with HCl, and extracted with the lower phase of $CHCl_3$–MeOH–H_2O (1:1:1). The extract was concentrated and the residue purified by preparative tlc on silica gel, eluting with $CHCl_3$–EtOAc (19:1). The major

band (detected with rhodamine B, R_f 0.6 ~ 0.7) was collected and gave 14.6 mg of **8** as a colorless oil: IR (neat film) 2829 (–OMe), 1096 cm^{-1} (C–O–C); ^{1}H-NMR ($CDCl_3$) δ 3.34 (s), 3.33 (s), 3.31 (s) (–OMe); mass spectrum *m/z* 712 (M^+).

Methylation of **4**. A mixture of 53 mg of **4** (dried *in vacuo* at 70° for ½ hr), 390 mg of NaH, and 0.5 mL of MeI in 5 mL of DMF (dried with type 4A molecular sieves) was stirred under Ar at room temp for 43 hr. Water and CH_2Cl_2 were added and the mixture neutralized with H_2SO_4 and concentrated *in vacuo* to remove excess MeI. The aqueous soln was washed with CH_2Cl_2, filtered, concentrated to remove traces of CH_2Cl_2, and applied to a 1.5 × 26-cm column of Diaion HP20AG in water. The column was washed with water until the effluent contained no detectable sulfate and then with MeOH. Concentration of the methanolic eluate gave 45 mg of **9**: IR (KBr) 1232 ($ROSO_3^-$); tlc (System A) R_f 0.31 (tr), 0.38 (w), 0.44 (tr), 0.47 (s).

Acid hydrolysis of **9**. Crude **9** (45 mg) was hydrolyzed in 0.1 N HCl and the product extracted as described for **5**, giving 28 mg of **10** as a white solid: tlc (System B) R_f 0.52 (w), 0.56 (w), 0.59 (m), 0.64 (m), 0.76 (s).

Tosylation of **10**. A soln of 28 mg of crude **10** and 107 mg of TsCl in 0.5 mL pyridine was left under Ar for 16 hr at room temp. The soln was cooled on ice, treated with a little water and after a few min mixed with 1 N HCl and extracted with ether. The extract was washed with 1 N HCl, sat $NaHCO_3$aq, sat NaClaq, dried ($MgSO_4$) and concentrated *in vacuo*, giving 36 mg residue. This was chromatographed on a Whatman PLK5F silica gel plate, eluting with $CHCl_3$. A fluorescence-quenching band, R_f 0.47 ~ 0.67, was collected, giving 20 mg. Rechromatography on a Merck silica gel 60 plate, eluting with benzene-ether, 19:1 (R_f 0.14 ~ 0.26) gave 15.6 mg of **11**: tlc (same system) R_f 0.25 (s), 0.30 (m), 0.33 (m), 0.37 (w), 0.45 (tr); IR (neat film) 1365, 1191, 1179 cm^{-1} ($ROSO_2Ar$); ^{1}H-NMR ($CDCl_3$) δ 7.76 & 7.33 (AB quartet, J = 8.5 Hz, ArH), 4.50 (m, H-C-OSO_2Ar), 3.32 & 3.22 (–OMe), 2.44 (ArMe), 1.24 [–$(CH_2)_n$–], 0.88 (RMe).

Lithium triethylborohydride reduction of **11**. A soln of 15.6 mg of **11** in 0.5 mL 1 M $LiEt_3BH$ in THF was kept under Ar at room temp for 64 hr. Water was added and the product was isolated with $CHCl_3$ to give 10.2 mg solid: tlc (benzene), R_f 0.02 (tr), 0.05 (tr), 0.27 (s), 0.47 (tr), 0.67 (m). Preparative tlc in this system and collection of a band with R_f 0.17 ~ 0.29 (detected with rhodamine B) gave 5.1 mg of **12** as a waxy, crystalline solid, m.p. 60 ~ 62°: tlc (benzene) R_f 0.29; ^{1}H-NMR ($CDCl_3$) δ 3.33 & 3.32 (–OMe), 1.26 [–$(CH_2)_n$–], 0.87 (RMe); IR (neat solid from melt) 1098 cm^{-1} (C–O–C); mass spectrum *m/z* 622 (M^+). (Found: C, 80.94; H, 13.77. Calc. for $C_{42}H_{86}O_2$: C, 80.95; H, 13.91%).

Acid hydrolysis of **2** *and trimethylsilylation of the hydrolysate*. A soln of 13.2 mg of **2** in 5 mL 0.1 N HCl was heated at 100° for 1.5 hr and the resulting mixture extracted with MeOH–$CHCl_3$ (1:1). Concentration of the extract gave 7.7 mg of solid: tlc (System A) R_f 0.42. Treatment with a mixture of bis-O,N-(trimethylsilyl)trifluoroacetamide and trimethylsilylimidazole gave the trimethylsilyl derivative **13**: mass spectrum *m/z* (relative intensity) 1012 (8), 1014 (7) (M^+ of **13a** and **13b**, respectively).

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